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(54) Title: Pt-CONTAINING COMPOUND, PROCESS FOR ITS PREPARATION, AND APPLICATION OF SUCH COMPOUNDS

(57) Abstract

Platinum complexes are disclosed which contain at least one leaving group and at least one detectable group. These complexes can be used for diagnostic purpose and for the labelling of nucleic acids. Specific example is Pt(ethylenediamine)(dimethyl sulfoxide)(fluorescein-NH{CS}-NHCH3).

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+ DESIGNATIONS OF "SU"

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Pt-containing compound, process for its preparation, and application of such compounds.

Such Pt-containing compounds are known from Reedijk, J. Struct. Bonding (Berlin), 67: 53-72.

This article describes the anti-tumor compound cis-$Pt(NH_3)_2Cl_2$, which compound has a high affinity for (amongst others) proteins and DNA molecules and particularly it appears that such a compound has a marked affinity for the N7-nitrogen atom in the purine bases Guanine and Adenine, as well as for sulphur groups in macromolecules.

By dissociation of the two chlorine ligands two reactive sites arise, with which such platinum compounds can cross-link between two neighbouring Guanine and/or Adenine bases in the same or in opposite DNA strands. The application of cis-platinum as anti-tumor drug (cytostaticum) is based on this mechanism.

Besides this related carbo-platinum compounds are known from the same literature, which also have a high affinity for amongst others proteins and DNA molecules in a similar way as cis-platinum compounds.

On the contrary monochlorinated platinum compounds like Pt(diene)Cl appear to keep their DNA affinity but they do not form cross-links and interfere only slightly with the base pairing of complementary DNA strands, and are as such not anti-tumor active.

According to U.S. patent specification No. 4,711,955 it is preferred to apply DNA/RNA technology in the present medical-biological practice, especially the diagnostical practice, when non-radioactive nucleic acid labelling techniques are available. The presently applied known non-radioactive labelling techniques for DNA and RNA are globally to be divided in two categories.

1. Labelling which proceeds via enzymatic or organic synthetic routes; for instance biotin, bromodeoxyuridine (BrdU), digoxygenine, fluorescein and peroxidase.

2. Labelling by direct chemical coupling, like photo-biotin, AAF, mercury, sulfone groups.

Application of such labels brings along a number of problems, which are particularly relat d to the complexity of the labelling procedure, the sometimes limited length of the synthetic oligonucleotides which are to be labelled, to use of health-injuring compounds and the stability of the label, when it is bound to the nucleic acid.

The invention now contemplates providing platinum-containing compounds, in the application of which the above-mentioned disadvantages are effectively removed.

To this end the invention provides a compound with the formula $\{Pt^{II}(w)(x)(y)(z)\}$ or $\{Pt^{IV}(u)(v)(w)(x)(y)(z)\}$ with the structural formula 1 or 2 respectively of the formula sheet, in which u, v, w, x, y and z represent whether or not the same whether or not interconnected ligands, from which at least one is a leaving ligand and at least one of the remaining ligands represents a detectable marker group.

Such a compound which is novel per se, and on the one side is provided with a directly or indirectly detectable marker group, as for instance a hapten, fluorescein or rhodamine and on the other side is provided with a suitable leaving group, is an especially suitable and novel DNA label with the general indication PtM (Pt stands for platinum and M stands for marker group) with unique properties.

For it appeared, that such a compound adheres spontaneously and irreversibly to DNA in aqueous medium. Further, the thus labelled DNA may be separated from the redundant compound with the formula 1 or 2 of the formula sheet by alcohol precipitation. An important advantage is, that the thus labelled DNA may be detected immediately after hybridization by means of a fluorescence microscope or indirectly with one of the known immunohistochemical staining techniques.

The advantages of the present platinum-containing compounds are shortly summarized:

1. Direct - almost instantaneous - labelling of macromolecules without necessity of enzymatic or organo-synthetic procedures.

2. One-step purification of labelled molecules by means of a simple routine technique.

3. Direct and/or indirect detection of labelled molecules by way of almost all known (microscopic) techniques.

As further advantage may be mentioned, that for specific purposes (for instance extra sensitive in situ hybridization of RNA) a radioactive (^{14}C or ^{35}S)-platinum-containing compound according to the invention may be applied as simple and fast (non-enzymatic) labelling of probes, followed by direct detection by means of autoradiography.

Another important new application of the probes labelled with the present compounds is in situ hybridization in the electron microscope whereby the high mass of the platinum atom in the compound according to the invention takes care for a direct probe-specific local increase of the electron density.

As leaving ligand $(CH_3)_2SO$, H_2O or Cl appears to be especially suitable according to the invention. It is observed that besides the just mentioned preferred leaving ligands in the compound with formula 1, respectively formula 2 of the formula sheet the following groups are qualifying, Br^-, I^- or F^-; SO_4^{2-}, NO_3^-, PO_4^{3-}, CO_3^{2-}, and analogues like ethylnitrate; phosphonates, oxalates, citrates and derivatives thereof; H_2O, ROH and RO^-, in which R is an organic residual group and substituted sulfoxides R^1R^2SO, in which R^1 and R^2 whether or not equal to each other, represent an organic residual group.

As the detectable marker group in the compounds with formula 1 or 2 of the formula sheet a fluorescent group generally merits the preference. A special preference merits fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC).

A very suitable compound according to the invention is {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)-$NHCH_3$)}, in the following abbreviated as PtF.

The novel compounds according to the invention are especially suitable for virus diagnostic purposes, bacteria diagnostic purposes, for detection of genetic deviations, detection of gene expression etc.

There are known a number of viruses, which cannot or with great difficulty be brought into culture, and of which

the serological diagnostical methods are extremely complicated, or which are very labile outside the body, and therefore unsuitable in contamination tests.

With some of these viruses the diagnosis may moreover be hindered by the necessity of differentiation between an acute stage of the illness, carriership, or virus genome insertion in the human DNA. In the meantime progress has been made herewith by using DNA probes. Some viruses have moreover serious pathogenic effects and are related with the development of malignant tumors. The accurate detection of these viruses and the correlation with a clinical follow-up of patients is therefore an important matter.

In principle virus strains or subtypes may be distinguished from each other by DNA/RNA probes.

Detection methods using labelled DNA or RNA probes appear to be able to solve these problems. Much progress has been made in the diagnosis of both DNA and RNA viruses. The advantage of these methods is that immediately the patient material (smears, samples of blister, nose and other fluids, tissue sections etc.) may be tested on the presence of virus DNA/RNA. Also retrospective studies have already provided important information about viral causes of mortality etc.

Further, the invention comprises a process for the preparation of Pt-containing compounds according to the invention with the formula $\{Pt^{II}(w)(x)(y)(z)\}$ or $\{Pt^{IV}(u)(v)(w)(x)(y)(z)\}$ with the structural formula 1 or 2 respectively of the formula sheet, in which u, v, w, x, y and z have the aforementioned meanings, characterized in that the Pt-containing compounds are prepared in a manner known per se for analogous compounds.

The preferred compound according to the invention, to wit PtF, is prepared by conversion of fluorescein-N=C=S with CH_3NH_2 in water, after which the mentioned fluorescein-NH(CS)-$NHCH_3$ is precipitated from the solution by acidifying to a pH of 2-3, after which the precipitate obtained is suspended in water and the pH of the suspension is brought to a value of 10-11 by addition of a base, providing a bright yellow solution, to which solution {Pt(ethylenediamine)-

(Me_2SO)Cl} in water is added and the reaction mixture is stirred at room temperature in the dark, after which the non-reacted fluorescein-NH(CS)$NHCH_3$ is precipitated by acidification and filtered and finally the filtrate is freeze-dried yielding {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)$NHCH_3$)} (PtF).

Subsequently, the invention extends to a diagnostic kit for use in the detection of viruses, bacteria, parasites, genetic deviations, gene expression, which kit comprises a Pt-containing compound according to the invention.

The invention is now further elucidated with reference to the following non-limitative examples.

Example I

Preparation of PtF for labelling purposes.

First of all fluorescein-NH(CS)$NHCH_3$ is prepared by reacting 100 mg fluorescein-N=C=S with 1 ml CH_3NH_2 in 100 ml water. The reaction takes about 1 hour under continuous stirring at room temperature in the dark. The obtained reaction product, fluor-escein-NH(CS)$NHCH_3$, is precipitated from the solution by acidifying with HCl (1 mol/liter {M}) to a pH of 2-3. The precipitate is washed in water and then collected.

Then a suspension of 100 mg (0.237 mmol) of the thus obtained fluorescein-NH(CS)$NHCH_3$ in 95 ml of water was brought with NaOH (1 M) on a pH of 10-11, whereby a bright yellow solution was obtained. To this solution was added 72 mg (0.178 mmol) of [Pt(ethylenediamine)(Me_2SO)Cl]Cl or [Pt(ethylenediamine)Cl_2]Cl in 5 ml of water and the reaction mixture was slowly stirred in the dark for 5-10 minutes at room temperature. The non-reacted fluorescein-NH-(CS)$NHCH_3$ was precipitated by acidification to a pH of 2-3 with HCl (1 M) and removed by filtration. The bright yellow filtrate was freeze-dried, yielding a stable dry compound {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)$NHCH_3$)}, or {Pt(ethylenediamine)Cl(fluorescein-NH(CS)$NHCH_3$)}, abbreviated PtF.

In principle the reaction may be carried out at an analogous manner with as starting material the one as mentioned above, provided that fluorescein is replaced by for instance rhodamine, AMCA, biotin, digoxygenin or any other hapten, which may be modified in such a manner that therein is present a double-bounded sulphur (S) atom, a -SR group, a NR'R'' group or a nitrogen ring (-N-), wherein R'R'' are equal or not equal to each other and represent an organic residual group. (Also H is possible). These S- or N-atoms serve as binding ligand for the platinum (Pt) atom.

Example II

Nucleic acid-labelling with PtF.

The dry PtF compound is dissolved at a concentration of 1 mg/ml in distilled water, which has been brought at a pH of 9-10 with NaOH.

Then DNA (single or double stranded) or RNA at an arbitrary concentration (for instance 100 µg/ml) was taken up in a low-salt buffer with a pH of about 8 (for instance 10 mM TRIS-HCl) and possibly fragmented by ultrasonication.

To the thus obtained nucleic acid solution a ten fold molar excess of PtF solution was added and after proper mixing the reaction mixture was incubated in the dark at room temperature for 30-60 minutes.

Next 1/10 volume part of a Na acetate (3M) solution of a pH of 5.6 was added to the reaction mixture and after mixing subsequently two parts of ethanol were added, after which it was thoroughly stirred and the reaction vial was then incubated for 15 minutes at 80°C or for 2 hours at -20°C.

The PtF-labelled nucleic acid was thereupon precipitated by centrifugation at 10.000 x g for 7 minutes. The obtained pellet was washed in 90% ethanol and the nucleic acid labelled with the PtF was dissolved at the desired concentration at an arbitrary buffer (for instance 10mM TRIS-HCl, a pH of 7.5, 0.3 mM EDTA).

The thus PtF-labelled nucleic acid is now ready for use.

Examples f the use f PtM-labelled nucleic acids:

Example III

Human papilloma virus cannot be cultured, but some subtypes (HPV 16/18) are positively connected with the origin of malignant tumors of amongst others the cervix and the penis.

By now labelling purified DNA of such a papilloma virus with PtM and then performing an in situ hybridization procedure on cells or tissue of for instance the cervix, the presence of the risk bearing type papilloma virus may be shown very specifically by means of a direct fluorescence procedure or an indirect immunohistochemical procedure with anti-PtM antibodies.

Example IV

a) Human papilloma virus cannot be cultured, but some subtypes (HPV 16/18) are positively connected with a large chance on the development of malignant tumors on cervix or penis. Further, probes are developed for amongst others the detection of DNA (Vaccinia, Herpes simplex (HSV1/2, Epstein Barr, and adenovirus) and RNA viruses (Rota virus, influenza A, Cocksackie B). Until present the diagnosis of acute infection with Hepatitis B virus is only possible by inoculation of chimpanzees (!), for the virus cannot be cultured in human cells.

b) Varicella zoster virus, too, is very difficult to culture: it lasts 5-14 days, before a culture may be assessed. Moreover the virus is very labile and may become inactivated during transport. A negative test is therefore no proof of absence of the illness. Over and above a VZV infection is on morphological grounds indistinguishable from infections with Herpes simplex virus. Even commercially available antisera do not give an answer in immunohistochemical tests.

c) Cytomegalo virus is very laboriously cultured; diagnoses within a week's time are impossible, within 6 weeks no exception. CMV infections form an important source of complications in transplant-patients and in patients with reduced defence (AIDS). A good monitoring of these patients is essential.

In the above-mentioned cases a, b and c, which figure as only some of the many possibilities of examples of virus diagnostics, diagnostics may be considerably simplified and accelerated by the application of hybridization techniques with PtM-labelled probes.

Example V

Bacteria diagnostics.

It appeared to be possible recently to detect also bacterial nucleic acids using DNA probes. Genes for bacterial toxins may be shown; however, it is not possible to discern whether these genes are expressed. Fast detection of chromosomal and plasmid coded virulence factors (amongst others Listeria monocytogenes, Clostridium perfringens enterotoxin, Vibrio cholerae enterotoxin, E. coli entero-toxins and invasivity, Shigella and Yersinia enterocolitica enteroinvasivity) are important applications in the diagnosis of food poisoning and the quality control in the food industry (end product control).

Detection of Helicobacter (formerly Campylobacter) pylori by DNA in situ hybridization with PtM probes in stomachbiopsies of patients with gastritis is well possible.

Also the DNA of Chlamydia trachomatis may be detected in for instance a sandwich assay, or by means of an in situ hybridization.

Example VI

Diagnostics of parasitic infections.

World-wide 2 millions of people pass away of malaria. In principle this can be prevented by timely correct diagnostics. The present (routine) microscopic methods are often all too complicated for third world countries. In the western world the difficult microscopic technique may be extended with in situ hybridization on routine preparations, using PtM probes. Through this differential diagnostics of malaria species is considerably simplified and can be carried out by minimally trained personnel. In the third world a dipstick test based on PtM is the appropriate route for fast and simple diagnostics.

As analogous examples may be valid infection illnesses caused by Schistosoma, Trypanosoma, toxoplasmas, etc.

Example VII

Detection of genetic deviations.

The hybridization technique with PtM probes offers the possibility for prenatal diagnostics of congenital deviations in for instance amniotic fluid punctates and chorionbioses. Postnatal detection of deviations (for instance malignities) is also possible, as well as extension of HLA typification for diagnosis of HLA associated illnesses.

Restriction fragment polymorfisms: Every human genome will fall apart, when treated with restriction enzymes, in a large number of specific fragments: the restriction fragments. If by a mutation the base sequence changes on a site where a restriction enzyme attacks, will this lead to the development of aberrant fragments. These fragments may be detected by suitable (PtM labelled) probes by means of DNA blotting methods (for instance in sickle cell anaemia, Duchenne muscle dystrophia, cystic fibrosis, Huntingdon chorea).

Immediate detection of aberrant DNA with synthetic oligonucleotide probes may take place when the base sequence belonging to a DNA deviation is known (ß-thalassemia, anti-thrombin III deficiency, grow hormones deficiency, haemophilia B, PKU .... etc.).

Detection of chromosome changes as translocations, deletes, inversions and duplications in the human karyotype may be detected by means of in situ hybridization followed by direct PtF fluorescence, or by Southern blotting of restriction fragments.

Example VIII

Detection of gene expression.

The visualization of the presence of a cellular antigen using immunochemical techniques does not prove that at that moment the relative gene are expressed. Neither does this indicate whether the shown product has an intra- or extracellular origin. Detection of mRNA within a cell gives

direct information about the expression of genes. This information may provide data on cell functioning, but may also be of assistance in diagnostics.

In view of the present problems for carrying out this RNA ISH (RISH) technique with non-radioactive probes, the application of the very direct PtM label is the appropriate way of performing such diagnostics because the problems particularly arise from the necessity to dispose of a well-penetrating immunohistochemical detection system. This last one may remain in abeyance with the application of direct PtM fluorescence.

Detection of deviating mRNA as a mark of heritable illnesses by means of blotting with radioactive cDNA probes has been proven to be possible already for a number of congenital deviations. The speed and applicability may be considerably increased here by non-radioactive (or radioactive) PtM labelling.

With PtM probes RISH or blotting may be applied in the diagnosis of cancer by means of detection of specific gene transcripts (for instance calcitonin mRNA in thyroid gland metastases, oncogene expression in malignant tumors), or the loss of germ line bands (loss of heterozygoty) or gene rearrangement (lymphomas).

CLAIMS

1. Pt-containing compound with the formula $\{Pt^{II}(w)(x)(y)(z)\}$ or $\{Pt^{IV}(u)(v)(w)(x)(y)(z)\}$, with the structural formula 1 or 2 respectively of the formula sheet, in which u, v, w, x, y and z represent whether or not the same whether or not interconnected ligands, of which at least one is a leaving ligand and at least one of the remaining ligands represents a detectable marker group.

2. Compound according to claim 1, **characterized in that** the leaving ligand is $(CH_3)_2SO$, Cl or H_2O.

3. Compound according to claim 1, **characterized in that** the detectable marker grouping is a fluorescent group.

4. Compound according to claim 3, **characterized in that** the fluorescent group represents fluorescein or tetramethyl rhodamine.

5. {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)-$NHCH_3$)} (PtF).

6. A process for the preparation of Pt-containing compounds according to one of the preceding claims, with the formula $\{Pt^{II}(w)(x)(y)(z)\}$ or $\{Pt^{IV}(u)(v)(w)(x)(y)(z)\}$, with the structural formula 1 or 2 respectively of the formula sheet, in which u, v, w, x, y and z have the meanings stated in claim 1, **characterized in that** the Pt-containing compounds are prepared in a manner known per se for analogous compounds.

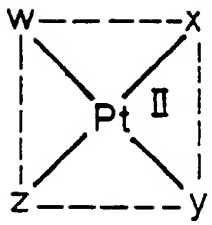
7. A process according to claim 6, **characterized in that** {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)-$NHCH_3$)} (PtF) is prepared, **characterized in that** fluorescein-N=C=S is converted with CH_3NH_2 in water, after which the said fluorescein-NH(CS)$NHCH_3$ is precipitated from the solution by acidifying to a pH of 2-3, after which the obtained precipitate is suspended in water and the pH of the suspension is adjusted to a value of 10-11 by addition of a base, yielding a bright yellow solution, to which solution {Pt-(ethylenediamine)(Me_2SO)Cl in water is added and the reaction mixture is stirred at room temperature in the dark, after which the non-reacted fluorescein-NH(CS)$NHCH_3$ is precipitated

by acidification and filtered and finally the filtrate is freeze-dried yielding {Pt(ethylenediamine)(Me_2SO)(fluorescein-NH(CS)-$NHCH_3$)} (PtF).

8. Application of the Pt-containing compound according to one of the preceding claims 1-5, for medical diagnostic purposes of virus, bacteria, or parasitic infection, detection of genetic deviations, detection of gene expression.

9. Diagnostic kit for use in detecting viruses, bacteria, genetic deviations, gene expression, which kit comprises a Pt-containing compound according to one of the preceding claims 1-5.

FORMULA



(1)
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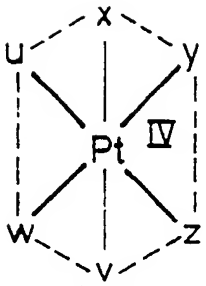
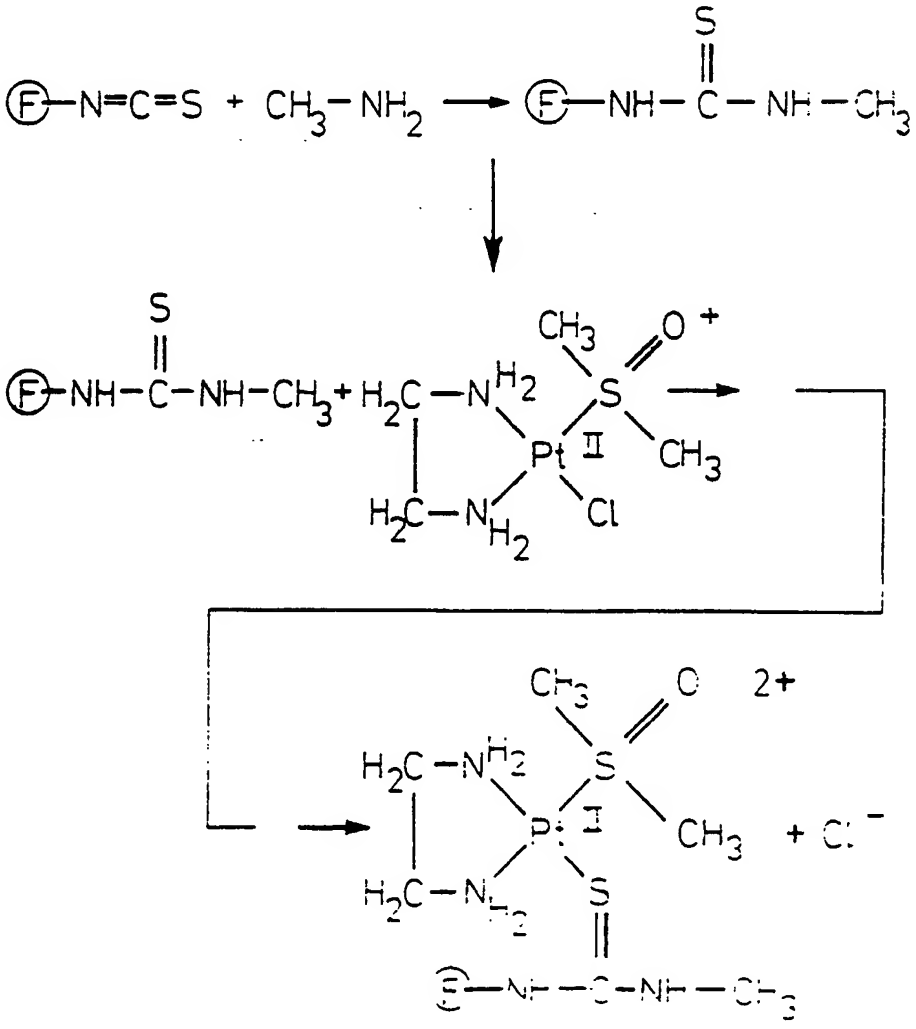


(2)
octahedral coordination

REACTION SCHEME



INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00126

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 F 15/00 G 01 N 33/00 G 01 N 33/52

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 F 15/00 G 01 N 33/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0186363 (JOHNSON MATTHEY INC.) 2 July 1986, see the whole document	1-3
A		4-7
X	US,A,4490543 (B.L. BERGQUIST) 25 December 1984, see the whole document	1-3
A		4-7
A	WO,A,8909598 (THE UNIVERSITY OF VERMONT AND STATE AGRICULTURAL COLLEGE) 19 October 1989, see claim 1	1-7

-/-

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26-09-1991	07 NOV 1991
International Searching Authority	**Signature of Authorized Officer**
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Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Canadian Journal of Chemistry, vol. 63, no. 9, September 1985, KG Campbell Corp., (CA), F.D. Rochon et al.: "Synthesis and nuclear magnetic resonance spectra of platinum compounds with thiourea derivatives", pages 2425-2429, see the whole article	1-7
A	EP,A,0147665 (MOLECULAR DIAGNOSTICS) 10 July 1985, see the whole document	8,9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9100126 SA 49712

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 31/10/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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